Use of D140 conditional-knockout cell lines to study chromosomal passenger protein function

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Use of DT40 conditional knockout cell lines to study chromosomal passenger proteins function

Xavier Fant[1], **Kumiko Samejima**[1], **Ana Carvalho**[1,4], **Hiromi Ogawa**[1], **Zhenjie Xu**[1,5], **Zuojun Yue**[1,3], **William C. Earnshaw**[1,*], and **Sandrine Ruchaud**[2,*]

[1]Wellcome Trust Centre *for* Cell Biology, University of Edinburgh, Edinburgh, EH9 3JR, UK

[2]CNRS USR3151, Station Biologique de Roscoff, place G. Teissier, 29680 Roscoff, FRANCE

[3]Cell & Developmental Biology, The University of Dundee, Dundee DD1 5EH, United Kingdom

[4]Ludwig Inst. for Cancer Research, Dept of Cellular & Molecular Medicine, 9500 Gilman Dr., La Jolla, CA 92093-0653, USA

[5]Department of Molecular and Cellular Physiology, Stanford University School of Medicine, 279 Campus Drive, Stanford, CA 94305-5345, USA

Abstract

The chromosomal passenger complex (CPC-INCENP, Aurora B kinase, Survivin and Borealin) is implicated in many mitotic processes. Here we describe how we generated DT40 conditional knockout cell lines for *incenp1* and *survivin1* to better understand the role of these CPC subunits in the control of Aurora B kinase activity. These lines enabled us to reassess current knowledge of Survivin function and to show that INCENP acts as a rheostat for Aurora B activity.

Keywords

DT40; Knockout; INCENP; Survivin

INTRODUCTION

Accurate chromosome segregation requires that kinetochores of sister chromatids bind microtubules that emanate from opposing spindle poles. During prometaphase, various aberrant kinetochore attachments occur which, if not corrected, can lead to improper chromosome segregation and aneuploidy. To avoid this, cells have a surveillance mechanism, called the spindle checkpoint, which delays anaphase until all sister kinetochores are properly captured and under tension. Kinetochore attachment and error correction are directly controlled by the Aurora B kinase, part of the chromosomal passenger complex (CPC).

In most organisms, the core CPC is composed of Aurora B kinase [1] and three non-enzymatic subunits, INCENP [2, 3], Survivin and Borealin/Dasra B [4-7] that control targeting, enzymatic activity and stability of Aurora B kinase [8]. The CPC controls many aspects of mitosis ranging from chromosome and spindle structure to the correction of kinetochore-microtubule attachment errors, regulation of mitotic progression and completion of cytokinesis [9]. Knockdown by RNA interference of any member of the complex disrupts

*authors for correspondence, sruchaud@sb-roscoff.fr or bill.earnshaw@ed.ac.uk.

NOTE Amino acid numbering corresponds to chicken proteins.

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mitotic progression [4, 7, 10-13]. Although INCENP has been shown to be responsible for the initial Aurora B activation through direct binding and a phosphorylation feedback loop [9, 14, 15], the mechanisms responsible for controlling the activity of this important kinase are still poorly understood.

The ability to shut down/off the expression of a gene by RNAi or knockout is widely used to study protein function in many eukaryotic systems. Whereas RNAi relies upon the destruction of the mRNA, which is continuously produced, conditional knockouts block production of the mRNA at its source. Another advantage of knockout strategies over RNAi is the absence of off-target effects [16]. Several cell types are used to generate knockouts including mouse ES cells, human RPE, Nalm-6, HT1080 and chicken DT40 cell lines [17-22].

DT40 cells are chicken B lymphoma with a very high rate of homologous recombination (up to 90% for some loci), greatly facilitating gene targeting. DT40s have been used to study many biological processes, including DNA damage pathways [23], transcriptional regulation [24], calcium signalling [25], apoptosis [26-29] and chromatin structure [30-32].

To better understand the role of the CPC in regulating Aurora B activity we studied the involvement of INCENP and Survivin by generating DT40 conditional knockout cell lines for their genes [33, 34].

1- Assessment of SURVIVIN function in mitosis and apoptosis

Survivin is a protein involved in mitotic progression and apoptotic regulation that is up-regulated in many human tumours. Survivin's expression peaks during mitosis [8, 35, 36]. The protein is required for targeting the CPC to centromeres [8, 37]. Survivin is considered a member of the inhibitor of apoptosis protein (IAP) family, despite lacking some of the key features shared by other members of the family. The exact role of Survivin in mitosis and apoptosis remains unclear.

Our lab generated two DT40 conditional knockouts of the *survivin* gene. We used this system to study the role of Survivin both in mitosis and apoptosis and to assess the role of key residues of the proteins in these biological processes.

1-1 KNOCKOUT STRATEGY

Survivin$^{-/-}$ cells were generated by deleting the entire *survivin1* gene (Acc. N°: ENSGALG00000008713, Figure 1A). Briefly, after targeting and replacement of the first allele by a resistance marker, the *survivin* cDNA, regulated by a Tet off system, was introduced in the heterozygous cells. The second allele was then targeted, generating a conditional Survivin knockout cell line which, upon addition of doxycycline exhibits the null phenotype. Stable transfection of this cell line with various Survivin cDNA-bearing mutations of interest allowed us to study their phenotypes in a null background.

1-2 THE ROLE of SURVIVIN in MITOSIS

Using our unique *survivin*$^{-/-}$ model, we confirmed some previous findings on Survivin function and revealed important features of key residues of the protein. For instance, we showed that Survivin is essential for both chromosome segregation and cytokinesis, concordant with observations from RNAi studies [12, 13]. A mutant previously shown to abolish interaction between Survivin and Aurora B (human Survivin$^{D70A/D71A}$, chicken Survivin$^{D72A/D73A}$) [38] introduced into the Survivin knockout cells was unable to concentrate normally at centromeres. This induced delocalization of the other passenger

proteins in early mitosis, surprisingly without affecting subsequent transfer of the CPC to the spindle midzone. Furthermore, this mutant showed a compromised spindle checkpoint.

Unexpectedly, despite the presence of these defects, this mutant could progress through mitosis and sustain cell growth, suggesting that Survivin binding to Aurora B is not absolutely required for cell survival [34]. In contrast with RNAi studies, we didn't observe any significant increase in defects in early phases of mitosis such as chromosome misalignment or abnormal spindles in the absence of survivin [12, 13, 39-41]. *Survivin*$^{-/-}$ cells exhibited mitotic arrest at high doses of taxol whereas at lower doses they could override the spindle checkpoint and progress through mitosis. This override was concomitant with increased apoptosis suggesting abnormal mitotic exit and subsequent cell death occurring in interphase [34].

1-3 THE ROLE of SURVIVIN in CELL DEATH

Similarly to what we observed for mitosis, *survivin*$^{-/-}$ cells enabled us to confirm some previously described results regarding Survivin's role in apoptosis and to contradict others. For instance we could confirm that some mutations in specific domains of Survivin did induce a loss of function phenotype, such as point mutations in the BIR domain (Survivin^{C59A} or Survivin^{C86A}) [42]. On the other hand, loss of Survivin did not induce an increased sensitivity to pro-apoptotic stimuli. Furthermore, abolition of Survivin binding to Smac (Survivin^{D55A}), an antagonist of Survivin involved in apoptosis, did not prevent cell growth nor did it display a pro-apoptotic phenotype, contradicting previous reports about the protective role of Survivin in apoptosis [42-46].

1-4 CONCLUSIONS

Generation of DT40 *survivin*$^{-/-}$ cells expressing various mutants, allowed us to reassess Survivin functions both in mitosis and in apoptosis in complete absence of the endogenous wild-type protein. The null background proved to be essential to study Survivin function. Discrepancies observed between our study and previous reports demonstrate the importance of a genetically-clean system to dissect the protein functions. Our study ultimately proved that cell death induced by loss of Survivin is linked to cell cycle defects (primarily a failure in cytokinesis) rather than disregulation of apoptosis.

2- Functionnal analysis of INCENP-Aurora B interactions

INCENP is the scaffolding protein of the CPC [2, 3]. Its N-terminus is required for centromere targeting of the complex whereas its C-terminus contains the IN box where Aurora B binds and gets activated. To study in more detail the interactions between INCENP and Aurora B, we generated a conditional DT40 knockout for *incenp1* (Acc. N°: ENSGALG00000007537), expressing INCENP bearing specific point mutations in the IN box. We chose residues predicted to be important for activation (W766) or binding of Aurora B (F802) to INCENP [15].

2-1 KNOCKOUT STRATEGY

To generate an *incenp1* knockout, we used a promoter hi-jack strategy (Figure 1B) [33, 47]. Here, the first allele was placed under the control of a Tet off system, which was achieved by replacing the endogenous promoter with a minimal CMV/TetO promoter, together with stable expression of the tTA/VP16 transactivator under the control of the *kif4A* promoter. The other *incenp1* allele had its ORF disrupted, potentially allowing expression of only the 28 first amino acids of the protein. In these cells, the addition of doxycycline shuts off expression from the endogenous *incenp1* allele (Figure 2A, B). This strategy enabled the

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conditional expression of the multiple spliced isoforms of INCENP (class I and class II) [48].

2-2 Regulation of Aurora B activity through INCENP binding

We used this system to study how interactions between INCENP and Aurora B affect the activity of the kinase along with CPC function and localisation. The two mutants generated had different binding capacity to Aurora B leading to different levels of kinase activity. Expression of INCENP^{W766G} in absence of wild-type INCENP abolished Aurora B interaction with INCENP, reducing Aurora B activity by 70%. INCENP^{F802A} mutant bound Aurora B in a normal fashion but kinase activity was reduced by 50% contrasting with predictions from previous structural analysis [15]. Expression of either INCENP^{W766G} or INCENP^{F802A} in a null background induced cytokinesis failure and rapid cell death [33].

Both INCENP^{W766G} and INCENP^{F802A} were properly localised at centromeres in early mitosis but failed to transfer to the spindle midzone in anaphase. We could thus demonstrate that mitotic entry and sister chromatid separation are independent of CPC formation or Aurora B activity whereas both are required for proper transfer of the CPC to the spindle midzone and completion of cytokinesis [33]. Despite reports from RNAi experiments showing that Aurora B activity is required for outer kinetochore assembly [49, 50], we were able to detect CENP-A, -H, -O, -T, -E and the Hec1/Ndc80 complex at kinetochores of prometaphase *incenp1*$^{-/-}$ cells suggesting that kinetochore assembly is not affected by the lack of CPC at centromeres [33]. Moreover, CENP-E and PRC-1 localisation to the spindle midzone of *incenp1*$^{-/-}$ anaphase cells demonstrated that a spindle midzone was present despite the absence of CPC [33].

Expressing INCENP mutants in a null background enabled us to analyse the relationship between Aurora B kinase activity and the spindle checkpoint response induced by taxol. We established that various Aurora B activity levels yielded different spindle checkpoint responses when cells were treated with low doses of taxol. A minimal Aurora B activity (~50%, INCENP^{F802A}) was sufficient to trigger a checkpoint response whereas lower activity (~30%, INCENP^{W766G} or 15% with Aurora B inhibitor) only induced a weak checkpoint response [33]. This probably reflects the ability of the kinase to correct kinetochore-microtubules mis-attachments.

2-3 CONCLUSION

We mutated residues of INCENP that were predicted by crystallography studies to affect Aurora B binding or activity. Using *incenp1*$^{-/-}$ cells, we could assess the in vivo effects of mutating these residues. We were able to show that modulation of Aurora B activity regulates CPC localisation and function during mitosis. Ultimately, the use of DT40 *incenp1*$^{-/-}$ cells expressing various mutants allowed us to strengthen the model in which INCENP acts as a rheostat for Aurora B activity [33].

3- CONCLUSIONS

The DT40 cell line is a powerful system to study protein function. The ability to perform conditional knockouts of essential, multiply-spliced cell cycle-regulated genes makes it an ideal system to screen for and characterise functional domains or residues through the analysis of specific mutants against a null background.

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ABBREVIATIONS

CPC	Chromosomal Passenger Complex
IAP	Inhibitor of Apoptosis Protein
ORF	Open Reading Frame
TetO	tet operator
tTA	Transactivator

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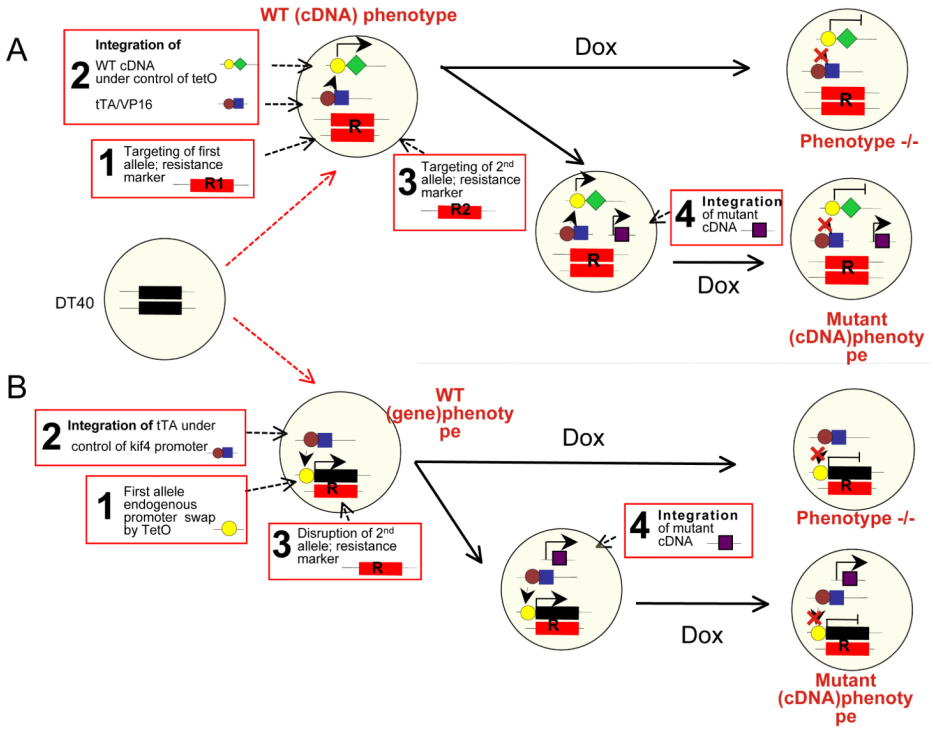

Figure 1. Schematic representation of the DT40 gene disruption and "conditional promoter"

A, Gene disruption strategy: The first *survivin1* allele is fully replaced with a resistance marker by homologous recombination (1). In a second step, a rescue construct containing, the transactivator (tTA/VP16) under control of either *kif4a* or CMV promoter and the Survivin cDNA under control of tet operator (TetO) are randomly integrated (2). Finally, the second *survivin1* allele is targeted and replaced by a resistance marker (3). Addition of doxycycline (dox) turns off the expression of the exogenous cDNA giving the null phenotype. cDNA encoding Survivin mutants are randomly integrated (4). Addition of doxycycline allows the observation of the mutant phenotype in a null background. **B, Conditionnal promoter strategy:** In a first step, the promoter of the first *incenp1* allele is swapped with a TetO by homologous recombination (1). The second step involves random integration of a construct containing the tTA under control of the *kif4a* promoter (2). In a third step, the second *incenp1* allele is disrupted by a resistance marker (3). The addition of doxycycline turns off the expression of the allele regulated by the TetO promoter giving the null phenotype. Random integration of wild type or mutant INCENP cDNAs (4) enables, after addition of doxycycline, the observation of their phenotype in a null background.

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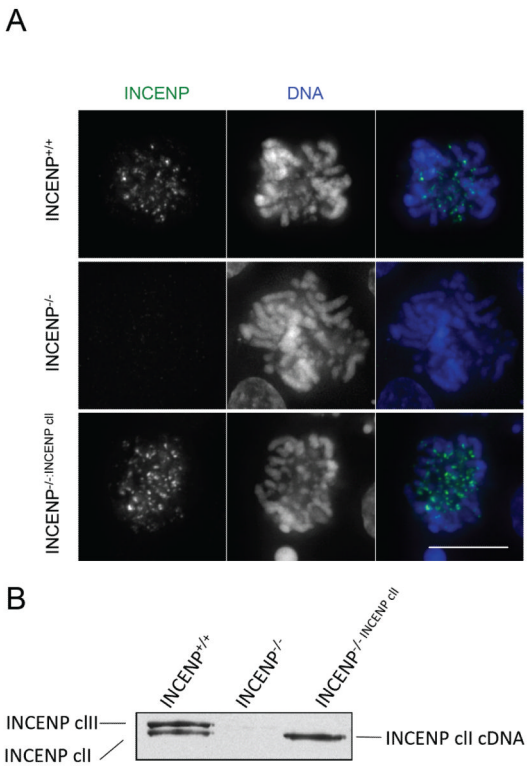

Figure 2. The conditional INCENP knockout model

A, Immunofluorescence of DT40 (*INCENP*$^{+/+}$), DT40 *INCENP*$^{-/-}$ or DT40 *INCENP*$^{-/-}$ expressing INCENP class I after 24h of doxycycline stained for INCENP (green) and DNA (blue). Bar 10 μm. **B,** Immunoblot comparing the levels of INCENP protein in *INCENP*$^{+/+}$ cells, *INCENP*$^{-/-}$ and in the *INCENP*$^{-/-}$ cells transfected with INCENP classI cDNA 24h after addition of doxycycline. Note the presence of two isoforms, class I and II in the wild type cells and the total absence of the protein in the knockout cells.